Common variation in the fibroblast growth factor receptor 2 gene is not associated with endometriosis risk

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BACKGROUND: Endometriosis is a polygenic disease with a complex and multifactorial aetiology that affects 8–10% of women of reproductive age. Epidemiological data support a link between endometriosis and cancers of the reproductive tract. Fibroblast growth factor receptor 2 (FGFR2) has recently been implicated in both endometrial and breast cancer. Our previous studies on endometriosis identified significant linkage to a novel susceptibility locus on chromosome 10q26 and the FGFR2 gene maps within this linkage region. We therefore hypothesized that variation in FGFR2 may contribute to the risk of endometriosis. METHODS: We genotyped 13 single nucleotide polymorphisms (SNPs) densely covering a 27 kb region within intron 2 of FGFR2 including two SNPs (rs2981582 and rs1219648) significantly associated with breast cancer and a total 40 tagSNPs across 150 kb of the FGFR2 gene. SNPs were genotyped in 958 endometriosis cases and 959 unrelated controls. RESULTS: We found no evidence for association between endometriosis and FGFR2 intron 2 SNPs or SNP haplotypes and no evidence for association between endometriosis and variation across the FGFR2 gene. CONCLUSIONS: Common variation in the breast-cancer implicated intron 2 and other highly plausible causative candidate regions of FGFR2 do not appear to be a major contributor to endometriosis susceptibility in our large Australian sample.

Keywords: endometriosis; fibroblast growth factor receptor 2; single nucleotide polymorphism; haplotype

Introduction

Endometriosis (MIM 131200) is a polygenic disease with a complex and multifactorial aetiology that affects 8–10% of women of reproductive age. There is extensive evidence that genetic variation influences disease susceptibility (Kennedy et al., 1995; Hadfield et al., 1997; Treloar et al., 1999; Simpson and Bischoff, 2002; Stefansson et al., 2002; Treloar et al., 2002). Endometriosis exhibits familial aggregation, being more common in the first-degree relatives of women with the disease than in the general population. Genetic factors account for 52% of the variation in liability to endometriosis and the relative recurrence risk to siblings was estimated at 2.34 in an Australian sample of twins (Treloar et al., 1999).

The reasons for establishment and progression of the disease remain uncertain. Epidemiological data support a link between endometriosis and cancers of the reproductive tract (Mostoufizadeh and Scully, 1980; Brinton et al., 1997; Fukunaga et al., 1997; Swiersz, 2002; Melin et al., 2006). Coexistence of endometriosis and breast cancer has been observed in several studies with relative risk factors ~1.3 among 20 686 women in Sweden who had been hospitalized with endometriosis (Brinton et al., 1997), and 1.08 among 63 630 women with endometriosis from the National Swedish Cancer Register (Melin et al., 2007). On the basis of a 31-year follow-up study, women with early diagnosed or long-standing endometriosis have a higher risk of ovarian cancer, with standardized incidence ratios of 2.01 and 2.23, respectively (Melin et al., 2006). Similarly, endometriosis appears to be commonly associated with endometrioid carcinomas and nearly 75% of the tumors arose in the ovary (Mostoufizadeh and Scully, 1980; Heaps et al., 1990; Fukunaga et al., 1997).

The fibroblast growth factor (FGF) family of signaling molecules is comprised of four membrane-spanning tyrosine kinase receptors and their alternatively spliced isoforms and 18 ligands (FGF1–10, FGF16–23) (Ornitz and Itoh 2001). FGF receptor 2 (FGFR2, OMIM 176943) has two isoforms resulting from tissue specific alternative splicing in the ligand binding domain; FGFR2b incorporating exon 8 is expressed in epithelial tissues and FGFR2c incorporating exon 9 is
expressed in mesenchymal tissues. The two isoforms demonstrate different ligand specificities which influence the redundancy and specificity of ligand binding and signaling (Ibrahimi et al., 2004). Combinations of FGFRs, FGFR isoforms and adaptor proteins comprise complex signaling networks that play crucial roles in the regulation of cell functions, such as proliferation, differentiation, migration and apoptosis (Taniguchi et al., 2000; Dmowski et al., 2001; Eswarukumar et al., 2005).

Cancer initiation and/or development are modified by dysregulation of growth factor signaling. Recently, FGFR2 has been implicated in both endometrial and breast cancer. Gain of function mutations in FGFR2 were identified in 15% of endometrial cancers demonstrating an endometrioid histology (Pollock et al., 2007). In normal human endometrial epithelium, FGFR2 mRNA and its protein are highly expressed (Siegfried et al., 1995; Moller et al., 2001), and increased FGFR2 expression in endometrial adenocarcinomas has been observed with developing stage of the tumor (Pekonen et al., 1999; Taniguchi et al., 2000). Stimulation with the inflammatory cytokine interleukin (IL)-1 up-regulated FGFR2 expression in human endometrial stromal fibroblasts (Li and Rinehart, 1998), suggesting there may also be inflammation related gene interactions in human endometriosis (Ness and Modugno, 2006). More recently, two genome-wide association studies in breast cancer have successfully identified and replicated associations of common genetic variants in the FGFR2 gene with this disease (Easton et al., 2007; Hunter et al., 2007). These studies were performed in large patient cohorts with validation observed in over 20,000 cases and controls (Easton et al., 2007; Hunter et al., 2007). In both studies, the most significantly associated SNPs were identified in intron 2 of FGFR2 and current studies are underway to try and identify the functional variant. It should be noted that FGFR2 has also been implicated as a tumor suppressor gene in several cancers where loss of expression has been associated with disease progression. Moreover, reintroduction of FGFR2 in several cell types has shown decreased growth and tumorigenicity including bladder (Ricol et al., 1999), salivary adenocarcinoma cells (Zhang et al., 2001), prostate (Yasumoto et al., 2004) and thyroid (Kondo et al., 2007).

The FGFR2 gene lies within a region of significant linkage to endometriosis on chromosome 10q. A combined linkage scan in 1176 Australian (n = 958) and UK (n = 218) families of sister pairs with surgically diagnosed disease identified a region of significant linkage on chromosome 10q26 (Treloar et al., 2005). The peak linkage signal was located at 148.75cM between markers D10S587 and D10S1656 and the 95% confidence interval (CI) spans a region of 8.5 megabase pairs (Mb). FGFR2 is located on chromosome 10q26 at 123.2 Mb, within the 95% confidence region for our linkage peak (119.4–127.9 Mb).

Variation in FGFR2 may represent a common pathway for both endometriosis and cancers of the reproductive tract. We hypothesized that variation in the FGFR2 gene could contribute to the genetic risk of endometriosis and may account for the linkage signal on chromosome 10q. We therefore conducted a case–control study to test for association between common variants in FGFR2 and endometriosis.

Materials and Methods

Participants and sample collection

The project was approved by the Human Research Ethics Committee of the Queensland Institute of Medical Research and the Australian Twin Registry. Women with surgically confirmed endometriosis were selected from each of our 958 Australian families as previously described (Zhao et al., 2006). The woman with the most severe stage of disease was chosen. Disease severity was assessed retrospectively from medical records using the revised American Fertility Society (rAFS) classification system (The American Fertility Society, 1985). Fifty-nine percent of cases were classified with minimal to mild endometriosis (rAFS stages I/II). The remaining 41% of cases with moderate to severe (rAFS stages III/IV) endometriosis were more likely to have ovarian endometriosis.

The controls were 959 unrelated women who had volunteered for a twin study of gynecological health (Treloar et al., 1999). Controls were selected after consideration of the competing issues of ascertainment bias from clinic controls and presence of undiagnosed cases (Zondervan et al., 2002). They were selected from women who self-reported they had never been diagnosed with endometriosis and were therefore considered to be at low risk of having endometriosis. Twins had been asked simply ‘have you had endometriosis?’ (Treloar et al., 1999). Additional information from medical records was used where available. Women were also asked whether they had ever had a laparoscopy and/or a hysterectomy and the reasons for each. About 27% of control women reported having a hysterectomy and/or laparoscopy. No evidence of endometriosis was reported at any of these procedures in our control sample (Zhao et al., 2007). The mean ages (±SD) of the cases and controls at the time of data collection were 35.82 ± 8.87 years (range = 17–65) and 45.60 ± 11.98 years (range = 29–90) years, respectively. Ethics approval was obtained from the Human Research Ethics Committee of the Queensland Institute of Medical Research and the Australian Twin Registry. Genomic DNAs were extracted (Miller et al., 1988), and diluted to a working concentration of 2.5 ng/µL. The case and control DNAs were randomly placed in 384-well PCR plates.

SNP selection

The SNPs across the FGFR2 gene were selected based upon the breast cancer allelic association results (Easton et al., 2007; Hunter et al., 2007) and regional LD (linkage disequilibrium) structure via both haplotype blocks (htSNPs) and pairwise r² (tag SNP/statistically similar SNP) information observed in International HapMap Project data (http://www.hapmap.org/). Selecting tag SNPs will avoid typing redundant SNPs and maximize the probability that a causative mutation is tagged by at least one marker genotyped in the study. Five highly plausible candidate SNPs (rs2981582, rs1219648, rs2420946, rs2981579 and rs11200014) located within intron 2 of the FGFR2 gene were identified from the two breast cancer genome-wide association studies (Easton et al., 2007; Hunter et al., 2007). We selected tagging SNPs (r² > 0.8) across the FGFR2 gene region, including the five breast cancer SNPs in the tag selection from the HapMap Center d’Etude du Polymorphisme Humain population (CEU). We found rs2981582 to be in perfect LD with rs1219648 and rs2420946 (r² = 1.0) and in strong LD (r² > 0.92) with rs2981579 and rs11200014 in this region in the HapMap database. We therefore only selected rs2981582 and rs1219648 to include in this study. A total of 40 SNPs were selected and spanned a region
of 150 kb across the FGFR2 gene, including 13 SNPs completely covering 27 kb of the entire intron 2 region. The chosen FGFR2 SNP list comprised 2 promoter, 30 intronic, 2 intron/exon boundary, 1 exonic and 5 3’ untranslated region (3’UTR) SNPs. We also typed one additional SNP not in the FGFR2 gene: rs10510126 (chr10:124992475), reported to be associated with breast cancer (Hunter et al., 2007). The SNP is located on chromosome 10q26 at 124.9 Mb, within the 95% confidence region for our linkage peak (Treloar et al., 2005). All SNP sequences were downloaded from the Chip Bioinformatics database (http://snpper.chip.org/) and the sequences were cross checked in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and Sequenom RealSNP databases (https://www.realsnp.com/) before assay design.

Genotyping

Multiplex assays were designed for 40 SNPs across the FGFR2 gene using the Sequenom MassARRAY Assay Design software (version 3.1). SNPs were typed using iPLEX™ Gold chemistry and analyzed using a Sequenom MassARRAY Compact Mass Spectrometer (Sequenom Inc, San Diego, CA, USA). The 2.5 µl PCR reactions were performed in standard 384-well plates using 12.5 ng genomic DNA, 0.8 unit of polymerase (HotStarTaq, Qiagen, Valencia, CA, USA), 500 µmol of each dNTP, 1.625 mM of MgCl2 and 100 nmol of each PCR primer (Bioneer, Korea). PCR thermal cycling in an ABI-9700 instrument was 15 min at 94°C, followed by 45 cycles of 20 s at 94°C, 30 s at 56°C, 60 s at 72°C. To the completed PCR reaction, 1 µl containing 1.4 units Shrimp Alkaline Phosphatase was added and incubated for 40 min at 37°C followed by inactivation for 5 min at 85°C. A mixture of extension primers was tested to adjust the concentrations of extension primers to equilibrate signal-to-noise ratios in the matrix-assisted laser desorption/ionization time of flight mass spectrometry prior to use for extension reactions. The post-PCR reactions were performed in a final 5 µl of extension reaction containing 1 × of termination mix, 1 × of DNA polymerase and 570 nM to 1240 nM extension primers. A two-step 200 short cycles program was used for the iPLEX Gold reaction as described in our previously study (Zhao et al., 2006). The iPLEX Gold reaction products were desalted by diluting samples with 18 µl of water and adding 5 µl of resin (Sequenom). The products were spotted on a SpectroChip (Sequenom Inc), and data were processed and analyzed by MassARRAY TYPER 3.4 software (Sequenom Inc).

Statistical analysis

SNP genotypes were tested for departures from Hardy–Weinberg equilibrium (HWE) separately for cases and controls using the PLINK genetic analysis package (http://pngu.mgh.harvard.edu/~purcell/plink/). Departures from HWE often indicate technical problems with SNP assays. The PLINK program is a toolset for whole genome association and population-based linkage analyzes and was used to test for association between endometriosis and individual SNPs or combinations of SNPs (haplotypes). In addition to obtaining nominal P-values, 10 000 permutation tests were performed to obtain a region-wide empirical P-value for each SNP. This maintained the individual genotypes as a whole while the individual’s disease status was shuffled. The method preserves the correlation between SNPs (LD) while breaking the relation between disease status and the genotypes. The global significance level was derived from these permutation tests and values <0.05 were considered to be statistically significant. Pairwise LD, haplotype frequencies and blocks were determined by Haplovew version 4.0 (Barrett et al., 2005) using the default method of Gabriel et al. (2002). To further investigate nominally associated SNPs, we utilized marker data from our previous linkase scan to infer SNP genotypes for case relatives using the Merlin program (Middeldorp et al., 2007).

Results

We typed forty tagging SNPs spanning a region of 150 kb across the FGFR2 gene in 958 endometriosis cases and 959 unrelated controls. All SNPs were in HWE. The position of SNPs within the gene and patterns of LD are shown in Fig. 1. Minor allele frequencies for the 40 SNPs ranged from 0.040 to 0.499 in our control samples and 0.042 to 0.496 in our case samples (Table 1).

We did not find any evidence for association between endometriosis and the key FGFR2 SNPs (rs2981582 and rs1219648) in the intron 2 region significantly associated with breast cancer (P > 0.5, Table 1). Both SNPs have strong LD (r2 > 0.93) in our samples. Haplotype analyses on the 13 SNPs in the entire intron 2 region of FGFR2 identified a single haplotype block with six haplotypes at frequencies ranging from 7% to 28% in both case and control samples (Fig. 1b). Tests of association with the haplotypes and endometriosis indicate none were contributing to disease susceptibility.

There was nominal evidence for allelic association with endometriosis for the intron 6 SNP rs2912770 with an asymptotic pointwise P = 0.004 (Table 1). The common T allele is associated with endometriosis (case frequency = 0.764; control frequency = 0.724). However, the difference was not significant after correcting for multiple testing (empirical familywise P = 0.1082). If rs2912770 is associated with endometriosis, we would expect other SNPs correlated (in strong LD) with rs2912770 to also show evidence of association. We therefore searched for statistically similar SNPs (ssSNPs) within the flanking 15 Mb (7.5 Mb either side) of rs2912770 using the web based program ssSNPer (Nyholt, 2006). There were three ssSNPs (rs1047100, r2 = 0.814; rs2912762, r2 = 0.730; and rs2071616, r2 = 0.720) identified, but since HapMap data indicate complete LD (r2 = 1) between rs2912762 and rs2071616, we only typed rs2912762 in our samples. Analysis of LD between SNP rs2912770 and the two additional SNPs rs1047100 or rs2912762 confirmed they are strongly correlated in our sample. Both SNPs showed decreased evidence of association with endometriosis compared with rs2912770 (rs1047100 asymptotic pointwise P = 0.020; rs2912762 asymptotic pointwise P = 0.121) and were not significant after correcting for multiple testing. To further evaluate the association signal observed for this SNP, we performed analyses using inferred genotypes (Middeldorp et al., 2007) from our chromosome 10 genome-wide linkage data together with our observed genotyping data. Linkage (allele-sharing) analysis (non-parametric linkage-pairs statistic) of the observed plus inferred data set produced a single point logarithmic odds (LOD) score of 0.11 (P = 0.2) at rs2912770. Family-based analyses of the inferred genotypes for rs2912770 also did not provide any evidence for association with endometriosis (χ2 = 0.489, P = 0.484). Association analysis of the additional SNP rs10510126 (chr10:124992475) showed no significant difference between our cases and controls with an asymptotic pointwise
Haplotype analyses using sliding windows of two to five contiguous \textit{FGFR2} SNPs did not identify any evidence for association between \textit{FGFR2} and endometriosis. To investigate effects of disease stage, differences between \textit{FGFR2} allele frequencies on subsets of endometriosis patients and controls were analyzed (Table 2). Stratification of cases according to stage of disease (564 rAFS Stages I/II cases and 959 controls) produced the smallest pointwise $P$-value of 0.002 for SNP rs2912770, but the familywise result was non-significant ($P = 0.065$) after correction for multiple testing.

In the permutations for 394 cases diagnosed with stage III/IV and 959 controls, the smallest pointwise $P$-value was 0.004 for SNP rs6585740, but the familywise result was not significant ($P = 0.125$).

\textbf{Discussion}

Our results do not support an association between endometriosis and variation in the \textit{FGFR2} gene region. The \textit{FGFR2} gene is a strong candidate and located inside the 95\% CI of our linkage signal on chromosome 10q (Treloar \textit{et al.}, 2005). We selected \textit{FGFR2} for association testing because of the evidence of gain-of-function mutations in endometrial carcinomas (Pollock \textit{et al.}, 2007) and the reports of genome-wide

\begin{figure}
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\includegraphics[width=\textwidth]{figure1}
\caption{Variation in the human \textit{FGFR2} region (a) the genomic structure of the \textit{FGFR2} coding region showing the location of the forty SNPs genotyped in 958 endometriosis cases and 959 controls, (b) common haplotypes and association analysis with endometriosis and (c) linkage disequilibrium plot of SNP estimated as $r^2$ using Haploview (white, $r^2 = 0$; shades of grey, $0 < r^2 < 1$; black, $r^2 = 1$).}
\end{figure}

The recent genome-wide association studies successfully identified \textit{FGFR2} as a novel susceptibility gene for breast cancer (Easton \textit{et al.}, 2007; Hunter \textit{et al.}, 2007). The common polymorphisms associated with breast cancer in these studies are located in the intron 2 region of \textit{FGFR2}. Several epidemiological studies support a link between endometriosis and reproductive cancers and several risk factors are common to endometriosis and breast cancer (Mostoufizadeh and Scully 1980; Bertelsen \textit{et al.}, 2007; Melin \textit{et al.}, 2007). To determine whether such an association exists in endometriosis, we genotyped 40 \textit{FGFR2} gene SNPs, including 13 SNPs covering a 27 kb LD block within intron 2 of the gene. We tested two key SNPs implicated in breast cancer (rs2981582 and rs1219648) and observed them to be in strong LD ($r^2 > 0.93$) in our samples. These data are consistent with the HapMap data and the Hunter group association study (Hunter \textit{et al.}, 2007). After correction for multiple tests, we found no evidence for association between the tested \textit{FGFR2} gene variants and endometriosis. Furthermore, given the high density of intron 2 SNPs examined, it is unlikely that variants in intron 2 of \textit{FGFR2} gene are responsible for initiation and/or development of endometriosis in Australian women.

Mutations in the gene region of \textit{FGFR2} play causative roles for some clinical disorders, including craniosynostosis syndromes and chondrodysplasia syndromes (Passos-Bueno \textit{et al.}, 1999; Wilkie \textit{et al.}, 2002). In addition, gain-of-function mutations of the \textit{FGFR2} gene have been identified and implicated in endometrial carcinomas providing perhaps the most compelling link between \textit{FGFR2} signaling and tumorigenesis (Pollock \textit{et al.}, 2007). Although endometriosis is not considered a malignant disorder, it does share common characteristics with malignant changes (Mostoufizadeh and Scully, 1980; Seidman, 1996; Fukunaga \textit{et al.}, 1997; Swiersz,
As discussed earlier, the FGFR2 gene lies within a region of significant linkage to endometriosis on chromosome 10q26 (Treloar et al., 2005). Linkage analysis for marker D10S1483 which is within the FGFR2 gene produced a LOD of 0.54 (pointwise $P = 0.06$) in the same data set (endometriosis case families) and a LOD of 0.56 (pointwise $P = 0.05$) in the complete linkage cohort (data not shown). The entire genomic sequence of FGFR2 is 869.95 kb which includes 750 kb of 3'UTR region. We chose SNPs that gave good coverage of the FGFR2 gene region because the reports of genome-wide association studies identified common variants in intron 2 of FGFR2 associated with risk of breast cancer and the evidence of a major gain-of-function FGFR2 mutation in exon 7 of FGFR2 in endometrial carcinomas. We also hypothesized that SNPs that negatively affected the tight tissue-specific alternative splicing in the ligand binding region of FGFR2 might allow leaky expression of the inappropriate splice form thereby allowing the inappropriate establishment of an autocrine loop, albeit at low levels. We hypothesized that such SNPs may then be associated with endometriosis by providing a subtle growth advantage to cells from both the epithelial and stromal endometrial compartments.

Our sample has good power to detect gene associations of small to moderate effect as described previously (Zhao et al., 2006). It is possible that some asymptomatic cases may be present in the control group. However, the resulting potential loss of power for a disease with a prevalence of 8–10% (Mosk-vina et al., 2005) would be negligible and therefore would not affect the conclusions from this study. Moreover, any potential loss in power is reduced further because our control group was drawn from women with self-reports of no previous diagnosis of endometriosis. We found no evidence for association between FGFR2 SNPs and endometriosis in the Australian samples, and unconvincing evidence of association between endometriosis and the intron 6 SNP rs2912770, suggesting that if the risk of endometriosis is influenced by common variation in the FGFR2 gene region in this population, the effect

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*Revised American Fertility Society (see ‘Participants and sample collection’ section).

Minor allele frequency.
size would be small. It has been observed that micro-RNAs are complementary to 3' UTR sequence motifs that regulate mRNA stability and mediate negative post-transcriptional regulation (Jackson, 1993; Lai, 2002). A recent study found that aberrant hypermethylation in the epigenetic silencing of the FGFR2 gene is related to human gastric cancer (Park et al., 2007). Further work would be required to determine whether either of these mechanisms is associated with endometriosis risk.

Our results demonstrate that variation in the highly plausible candidate regions of FGFR2 do not explain the linkage to endometriosis previously reported for this region of chromosome 10 (Treloar et al., 2005). FGFR2 is a good candidate but the linkage region is broad (8.5 Mb) and ~50 genes are located within the 95% CI for the linkage peak. Although many of these other genes can also be considered candidates for endometriosis due to our limited knowledge of the underlying biological mechanisms contributing toward endometriosis, variation in or associated with any one (or more) of these 50 genes may explain the linkage signal in this region and contribute to the genetic susceptibility of endometriosis.

In conclusion, we examined association between endometriosis and individual common SNPs and haplotypes in the FGFR2 gene region in a large Australian population. Our data show no evidence for association between endometriosis and FGFR2 SNPs or haplotypes in our case–control study. We conclude it is unlikely that variants across the FGFR2 gene intron 2 and entire coding region are associated with endometriosis and there is no evidence that variants in the FGFR2 gene region account for the linkage signal on chromosome 10q. The finding also underlines the genetic complexity between endometriosis and female reproductive cancers.

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